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From clonal to sexual hybrids: genetic recombination via triploids in all-hybrid populations of water frogs

Running title: From clonal to sexual hybrid water frogs

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Speciation via interspecific hybrids is very rare in animals, as compared to plants. Whereas most plants overcome the problem of meiosis between different chromosome sets by tetraploidization, animal hybrids often escape hybrid sterility by clonal reproduction. This comes at the expense of genetic diversity and the ability to purge deleterious mutations. However, here we show that all-hybrid populations of diploid

(LR) and triploid (LLR and LRR) water frogs (*P. esculentus*) have secondarily acquired sexual reproduction. First, in a crossing experiment analyzed with microsatellite markers, triploid hybrids of both sexes and genotypes (LLR and LRR) recombined their homospecific genomes. Second, the great majority of natural populations investigated had low multilocus linkage disequilibrium, indicating a high recombination rate. As predicted from mating system models, the L genome had constant, low levels of linkage disequilibrium, while linkage disequilibrium in the R genome showed a significant reduction with increasing proportion of recombining triploids. This direct evidence of sexual reproduction in *P. esculentus* calls for a change of the conventional view of hybridogens as clonally reproducing diploids. Rather, hybridogens can be independent sexually reproducing units with an evolutionary potential.

Key words: polyploidy, hybridogenesis, microsatellites, *Pelophylax esculentus*, *Rana esculenta*

Hybridization instantly creates individuals with a new genetic composition and is therefore a potentially powerful force in evolution (Wissemann 2007; Jiggins et al. 2008). Whether hybridization leads to speciation depends on the hybrids' ability to survive and reproduce (Arnold and Hodges 1995; Barton 2001; Chapman and Burke 2007). Two reproductive challenges need to be overcome for establishment of new hybrid taxa: First, the hybrids must be fertile, in spite of having two dissimilar chromosome sets which might interrupt meiosis (Arnold and Hodges 1995; Chapman and Burke 2007). Second, the hybrids must be spatially or reproductively isolated from the parental species (Wang et al. 2001; James and Abbott 2005; Chapman and Burke 2007).

Normal meiosis, as well as reproductive isolation, can instantly be restored by tetraploidization. Via this process, hybridization has had a large impact on plant evolution

(Arnold 1997; Hegarty and Hiscock 2005; Wissemann 2007), while in animals, remarkably few examples of tetraploid speciation are known (Orr 1990; Otto and Whitton 2000). In animal hybrids, fertility and reproductive isolation are, however, often established by different kinds of clonal reproduction which may or may not be accompanied by polyploidy. Among clonal vertebrates, reptiles are parthenogenetic, while fishes and amphibians depend on sperm from a sexual species for initiating embryogenesis (Vrijenhoek et al. 1989): In gynogenetic taxa, the sperm activates, but usually does not fertilize the eggs. In hybridogenetic taxa, fertilization takes place; yet, there is normally no recombination between the parental genomes. This is because the paternal genome is usually excluded from the germ line prior to meiosis while the remaining maternal genome is transmitted clonally (Dawley 1989). Hybridity is restored each generation by matings with the paternal species. The hybrids' soma is thus made up by both the sexual paternal and the clonal maternal genome, while the hybrids' germ line contain only the latter.

For hybrid speciation to be of evolutionary importance, a third factor is crucial: genetic recombination. Genetic recombination via sexual reproduction enhances genetic diversity and is generally agreed to convey three important benefits: One, high genetic diversity is required for defence against fast evolving parasites (Red Queen hypotehsis, Hamilton 1980). Two, the combination of beneficial mutations from different individuals enhances the efficiency of selection (Fisher 1930; e.g. Colegrave 2002; Cooper 2007). Three, and most importantly, the combination of deleterious mutations allows their purging from the population (Muller 1932; e.g. Vrijenhoek 1994). Without recombination, clonal lines are predicted to accumulate deleterious mutations via Muller's ratchet, which will eventually lead to their extinction.

As a consequence of their clonal reproduction modes, parthenogenetic, gynogenetic and hybridogenetic hybrid animal taxa lack the above mentioned advantages of genetic diversity and the ability to purge mutations. Hence, they are generally considered to be

78 “evolutionary dead ends”, at least as far as individual lineages are concerned (e.g. Vrijenhoek
79 et al. 1989; Maynard Smith 1992). In agreement with this, strictly clonal taxa are, with very
80 few exceptions (Butlin 2002), distributed as short-lived tips on the tree of life mainly
81 comprised of sexual taxa (Simon et al. 2003).

82 However, at least genetic diversity seems to be higher in clonally reproducing taxa
83 than previously assumed, and various mechanisms have been described how this can be
84 achieved. First, clonal hybrids often arise recurrently from different progenitors. Hence, they
85 have a high genetic diversity possibly enabling them to fit different ecological niches (frozen
86 niche variation hypothesis, Vrijenhoek 1984). Recurrent origin of clonal and polyploid sexual
87 lineages is known from several plants (Soltis and Soltis 1999) and also from animals,
88 including ostracods (Little and Hebert 1997), fishes (Janko et al. 2003; Pala and Coelho
89 2005), reptiles (Moritz et al. 1989) and some anurans (Ptacek et al. 1994; Stöck et al. 2005).
90 Second, some allegedly asexual organisms are not strictly clonal but occasionally incorporate
91 new nuclear material from a sexual host (Hedges et al. 1992; Spolsky et al. 1992; Schartl et al.
92 1995). The most recent discovery of such a mechanism is “kleptogenesis” in unisexual
93 salamanders of the genus *Ambystoma* (Bogart et al. 2007): all-female lines can incorporate
94 (parts of) nuclear genomes from sperm from sympatric sexual species and presumably later
95 discard other parts of the genome. Third, in bisexual hybridogenetic species, like the edible
96 frog, *Pelophylax esculentus* (called *Rana esculenta* until Frost et al. 2006), and the Iberian
97 minnow, *Squalius alburnoides*, hybrid x hybrid matings lead to offspring with parental
98 genotype (cf. Fig. 1b). Although rarely viable, these offspring could recombine the otherwise
99 clonal genomes if they succeed in reproducing (Hotz et al. 1992; Alves et al. 1998; Vorburger
100 2001c). While the existence of these three mechanisms can not be denied, their potential for
101 lifting the doom of “evolutionary dead end” from the relevant hybrid taxa is subject to
102 discussion.

Here we investigate the potential for systematic and frequent sexual reproduction in hybridogens through a mechanism called meiotic hybridogenesis. The term refers to the possibility that in polyploid hybridogens of the general type AAB, the homospecific chromosome sets from one parental species, A, recombine in a normal meiosis, whereas the set from the other parental species, B, is discarded (Alves et al. 1998). Preferential pairing of homologous chromosomes and elimination of the unmatched chromosomes has been shown for a number of triploid fish and frog hybrids (see Morishima et al. 2008 and references therein) but, so far, clear evidence for recombination through meiotic hybridogenesis comes from one species only: the Iberian minnow, *S. alburnoides* (Crespo-Lopez et al. 2006).

It might be argued that meiotic hybridogenesis is a rare and special phenomenon without much general relevance for the role of hybrids in animal evolution. However, meiotic hybridogenesis is interesting as a newly discovered possibility for hybridogenetic hybrids to obtain recombination in a regular, non-accidental way. Besides, the list of taxa with meiotic hybridogenesis will surely grow: Firstly, with the increasing application of molecular tools to organisms from different populations, the list of known hybridogens has grown recently and is likely to grow further. Secondly, since hybridogenesis was originally discovered in the diploid topminnow, *Poeciliopsis monacha-lucida* (Schulz 1969), polyploidy in hybridogens increasingly appears to be the rule, rather than the exception. At present, polyploidy is known from four of the six genera with hybridogenesis. Water frogs (*Pelophylax*, Berger 1967), Iberian minnows (*Squalius*, Carmona et al. 1997), spined loaches (*Cobitis*, Saitoh et al. 2004) and Oriental weatherloaches (*Misgurnus*, Morishima et al. 2008) excibit polyploidy while only hybridogenetic topminnows (*Poeciliopsis*) and stick insects (*Bacillus*, Bullini and Nascetti 1990) are purely diploid. Polyploidy is also known from a hybridogenesis-related mode of reproduction in the Batura toad (*Bufo viridis* complex, Stöck et al. 2002). We are thus just at the beginning of discovering the diversity and implications of hybrid reproduction modes.

Hence, investigating the extent of recombination during meiotic hybridogenesis and thus the long-term evolutionary potential for intraspecific hybrids seems timely and potentially relevant for more species than presently assumed. The edible frog, *Pelophylax esculentus*, provides a particularly interesting system for such an investigation, because it is the only hybrid yet known also to form self-sustaining, hybridogenetic, all-hybrid populations. In the absence of the parental species, meiotic hybridogenesis is the sole potential source of frequent recombination and could thus be of crucial evolutionary importance for these populations. Moreover, *P. esculentus* comes in various mating systems and, hence, offers an opportunity to study successive stages of incipient hybrid speciation.

The *Pelophylax esculentus* systems

Pelophylax esculentus (*Rana esculenta*) originated, and still originates, from interspecific matings between the two sexual water frog species, *P. lessonae* (the pool frog, genotype LL) and *P. ridibundus* (the marsh frog, genotype RR). The parental species, as well as the diploid *P. esculentus* hybrid with the genomic composition LR, have wide distributions in Europe. In the western part of this distribution area, LR excludes the L genome from the germ line prior to meiosis and transmits the R genome to the gametes clonally. As a result, matings between hybrids yield RR offspring, but these typically die due to homozygosity for deleterious mutations in the clonal R genome (Vorburger 2001a and references therein; Guex et al. 2002). In order to form a new generation of hybrid LR, *P. esculentus* is dependent on L gametes obtained from mating with *P. lessonae* (LE system, Fig. 1). In parts of Eastern Europe the pattern is reversed: hybrid LR excludes the R genome, produces L gametes and, therefore, lives in sympatry and mates with *P. ridibundus* (RE system). In both of these diploid systems, (reviewed by Graf and Polls Pelaz 1989) *P. esculentus* face disadvantages with respect to both of its genomes: the one in the hybrid's germ line is clonal, while the other, sexual, genome must for every generation be obtained by mating with the parental species. Various LE, RE

155 systems and *lessonae-esculentus-ridibundus* populations with both diploid and triploid *P.*
156 *esculentus* also exist (Günther 1991; Tunner and Heppich-Tunner 1992; Rybacki and Berger
157 2001), but unfortunately hardly anything is known about how these diverse and complicated
158 populations function.

159 The present study focuses on all-hybrid populations of *P. esculentus* (EE system) that,
160 by definition, live and reproduce without any of the parental species. Thus, the propagation of
161 both parental genomes, as well as any recombination within them, must be undertaken by
162 hybrids alone. All-hybrid populations are found in large areas of Denmark, southern Sweden
163 north-eastern Germany, and patchily in northern Poland and probably a few localities in south
164 eastern Europe (Mikulícek and Kotlík 2001; Rybacki and Berger 2001; Christiansen et al.
165 2005; Arioli 2007 cap. 5); (reviewed by Plötner 2005). These populations consist of diploid
166 (LR) and one or two types of triploid hybrids (LLR and LRR). LLR frogs of both sexes
167 provide L gametes while LRR make R gametes. Within the diploid LR, all males and some
168 females produce R gametes, while all females and a few males make unreduced LR gametes
169 yielding new triploids upon fusion with haploid gametes (Graf and Polls Pelaz 1989;
170 Christiansen et al. 2005; Arioli 2007 cap. 1; Jakob 2007 cap. 5). Sex determination is an xx-
171 xy system with a dominant male-determining y factor. The y factor is supposed to be present
172 in the L genome only (Graf and Polls Pelaz 1989; Berger and Günther 1991-1992), which
173 means that L genomes are either L_x or L_y while all R genomes are R_x. As a consequence, LLR
174 and LR come in both sexes, while the great majority of LRR are females (Jakob 2007 cap. 2
175 and the present study). In this way, the mix of di- and triploid hybrid frogs form self-
176 sustaining populations producing all gametes needed for a new generation of similar
177 composition (Fig. 1). Non-hybrid LL and RR offspring are also formed, but die off in natural
178 ponds during the tadpole stage (Arioli 2007 cap. 3).

179 In all three systems, clonally propagated *P. esculentus* genomes face the risk of
180 mutation accumulation. In the LE and RE systems, some accumulation can be tolerated, as the

clonal genome is constantly paired with a healthy parental genome in the hemiclonal hybrids (confirmed in LE by Vorburger 2001b). Nevertheless, the lifespan of the clonal genomes in diploid systems appears limited, as old clones are likely to become inviable or replaced by new genomes that were more recently derived from primary hybridization between the parental species. In the all-hybrid populations, the situation was, so far, unknown. It was often assumed that the LLR recombine their two L chromosome sets after exclusion of the R, and that, likewise, LRR recombine their two R sets after exclusion of the L genome (Günther et al. 1979; Graf and Polls Pelaz 1989; Som and Reyer 2006a). Under this assumption, the all-hybrid populations might be functionally sexual with a higher evolutionary potential than diploid LE and RE system populations. However, experimental evidence for recombination in triploids is scarce and controversial, due to low availability of polymorphic genetic markers. Based on allozyme and sex data, Günther et al. (1979) probably found recombination in one Polish LRR male (table 5, cross 25/26). Furthermore, Arioli (2007 cap. 1), using microsatellite analysis on Swedish frogs, detected recombination in an LRR female, but not in an LLR male. While these data demonstrate the capability of triploids to recombine, it remains unclear whether recombination happens as a rule or as an exception and whether there are sex- and/or genotype- (LLR vs. LRR) specific differences in the recombination rate.

Here we present the first crossing experiment with a sufficient number of frogs (30) and polymorphic genetic markers (18) to conclude that intragenomic recombination takes place in triploids of both sexes and genotypes (LLR and LRR). We also provide previously unpublished microsatellite primers and new multiplex PCR protocols.

Confirming recombination in triploids does, however, not suffice to conclude that all-hybrid populations are functionally sexual. Therefore, assessment of the impact of triploid-mediated recombination on the genetic structure of the L and R genomes in wild populations was needed. One might expect populations with many triploids to be highly recombined and thus have low multilocus linkage disequilibrium. This, however, should be true only for the R

genome; not for the L genome. The reason for the difference is that R gametes can originate from both recombining LRR and non-recombining LR frogs whereas L gametes come from recombining LLR frogs alone (see Fig. 1). The monopoly on L gamete production guarantees LLR frogs a large and constant reproductive contribution to the next generation and, hence, should result in high recombination rates of L genomes, irrespective of the LLR/(LLR+LR) ratio. This prediction was previously confirmed by a mathematical model (Som and Reyer 2006a), but empirical data are lacking. In contrast, recombination rates of R genomes should, on average, be lower but increase with LRR/(LRR+LR) ratios. For this prediction, neither theoretical nor empirical studies were available.

Here we show that linkage disequilibrium was low in a large sample of natural populations from across the Danish and Swedish range, indicating that natural recombination rates are sufficiently high for these all-hybrid populations to be functionally sexual. We also provide evidence for the expected correlations between linkage disequilibrium and population structure. Finally, we confirm that pond-specific influences and method-specific biases were without importance for these results. In conclusion, the all-hybrid populations are an example of a hybridogen that, in a unique way, has become an independent evolutionary unit with sexual reproduction and thus a long-term evolutionary potential.

Methods

Overview

The study was carried out on Swedish and Danish all-hybrid populations, because these are geographically isolated from populations with parental species (Christiansen et al. 2005; Jakob 2007 cap. 2).

For direct evidence of whether triploids recombine, adult frogs were sampled, genotyped and crossed and the offspring were reared and genotyped. Then, segregation and linkage analyses were performed on the inheritance pattern of the microsatellite alleles

analyzed. Absence of linkage between the majority of loci, when compared pairwise, would indicate recombination.

For investigating the level of recombination in natural populations, frogs were sampled in ponds with different proportions of clone-propagating diploid (LR) and recombining triploid frogs (LLR and LRR). All individuals were genotyped, and the multilocus linkage disequilibria in the L and R genomes were calculated as $\bar{r}_d$ for each pond separately. Low $\bar{r}_d$ values would indicate high levels of recombination. The effects of genome, population structure, pond-specific effects and method-specific biases on $\bar{r}_d$ were investigated to test the predictions outlined in the introduction and to test suspicions of artifacts. Finally, F statistics were calculated, because non-random mating, resulting in high F_{IS} values, would also affect $\bar{r}_d$.

Crosses

Genetic variation in Swedish and Danish *P. esculentus* is very low (Christiansen et al. 2005; Arioli 2007 cap. 4). To obtain genetic data at multiple heterozygous loci for linkage analysis, it was therefore necessary to: 1) Screen both published and unpublished microsatellites for polymorphism in Scandinavia and design multiplex PCRs with the final selection of 18 primer pairs. 2) Select the most heterozygous triploids of a large sample of frogs for crossing. 3) Raise the larvae to an age where offspring genotypes could be inferred reliably when the heterozygous parents shared an allele. When parents share one allele, alleles or even whole chromosomes missing in the offspring can lead to misinterpretation of the parental contributions. This was of real concern, because many young larvae are aneuploid, i.e. they have mixed, uninterpretable genotypes with extra or missing alleles (Christiansen et al. 2005 and unpublished data from the present study). Raising the larvae to metamorphosis ensured that most aneuploid offspring died off and did not enter the analyses.

Crossing and rearing took place at Stensoffa Field Station, Scania, Sweden. Between May 12 and 22, 2006, i.e. after their emergence from hibernation and before breeding, 269 frogs were caught at night using flashlight and dip net in one of the Danish (Alsønderup in Christiansen et al. 2005) and 10 of the Swedish ponds included in the investigation of natural populations described below. The frogs were marked individually with a transponder (Trovan ID101, Euro I.D., DE), toe-clipped for DNA analysis, and kept at approximately 7° C while the DNA samples were sent to the University of Zürich and analyzed for genome composition (LLR, LR, LRR) and heterozygosity (at LL in LLR and RR in LRR). The triploids with most heterozygous loci were preferred for the crossings, because recombination can only be assessed from combinations of heterozygous loci. This preference made a balanced design of source ponds impossible. Since males were more common than females among LLR frogs and females were predominant among LRR frogs, LLR males and LRR females were picked from a larger sample and were therefore more heterozygous than LLR females and LRR males. Most L genome data therefore derived from males and most R genome data from females.

Six crossing tables were designed, each having 3-4 females and 5-6 males including at least one LLR, LR and LRR female and at least one LLR, two LR and one LRR male. Substitute frogs were added if the sperm or egg quality looked suboptimal. All females were crossed with all males within the same crossing table, so that all frogs were crossed to all genotypes (half-sib design).

Offspring were produced on May 30, 2006 by artificial fertilization as described by Berger *et al.* (1994). Sperm solutions from the testes of hormone-injected males were distributed into 3-5 petri dishes per male. Eggs were then gently squeezed out of the hormone-treated females and dropped directly into the individual sperm solutions of the 5-6 different males, in small portions and in random order. The following day, the egg clumps were transferred to 1 liter tubs with 1-2 cm of water and subdivided for better oxygen supply.

The water was changed every 2-4 days and the egg jelly was removed after hatching. On June 12, when most tadpoles had just reached the feeding stage, 15 healthy-looking tadpoles (or fewer, if 15 were not available) from each sibship were randomly selected for rearing in 40 liters outdoors tubs covered with mesh lids allowing air and sunlight through, but keeping predators out. Algae growing on the insides of the tubs, supplemented with rodent pellets, ensured food *ad libitum*. Filamentous algae were regularly removed, fowling water exchanged and Daphnia *sp*. added for good water quality. The tadpoles metamorphosed from July 18 onwards. Slow-growing tadpoles were eventually moved indoors into smaller tubs, where the last ones metamorphosed in mid October. Offspring that died early during rearing disappeared, while offspring that died as metamorphs or nearly metamorphosing tadpoles were attempted DNA-analyzed although they were sometimes rotten. In total, 1628 tadpoles were selected for rearing, DNA samples were obtained from 1487 offspring (91%), and 1463 offspring (90%) were successfully genotyped.

Natural populations

Population structure was investigated in 54 Danish and 12 Swedish ponds from mid May to mid August 2005. The Danish ponds were chosen as pairs of ecologically distinct ponds, maximally 5 km apart, from across the area of distribution. At each location, approximately 30 frogs (predominately adults) were caught at night with flashlight and dip net, were measured and had a toe tip cut for DNA analysis before being returned to their pond.

The Swedish ponds constituted 11 ecologically variable ponds in the center of the small distribution area in Scania, Southern Sweden, and one from a satellite population near Malmö, 18 km west of the others ("core ponds" in Jakob 2007 cap. 2). The Swedish ponds were sampled as described above, but in both May and August, and the frogs were additionally marked with a transponder for individual identification. The Swedish samples are thus the sum of different individuals from the two catching rounds.

In total, 2296 Danish and Swedish frogs were caught and genotyped.

Laboratory protocols

DNA from the ethanol-stored toe-tips was extracted with Qiagen BioSprint 96 DNA Blood Kit following Qiagen's protocol for tissue extraction. All samples were subjected to two PCRs with nine primer pairs each. The reactions were of 5 µl and contained 0.8 µl DNA extraction, 2.5 µl Qiagen Multiplex PCR Master mix and 1.7 µl primer mix. PCR 1 contained primers Res16, Res20 (Zeisset et al. 2000), RlCA5, RlCA1b5 (Garner et al. 2000), Ca1b6, Ga1a19, Re2CAGA3 (Arioli 2007 cap. 4), RlCA2a34 and Rrid064A (table 1). PCR 2 contained Res22 (Zeisset et al. 2000), RlCA18 (Garner et al. 2000), Rrid013A (Hotz et al. 2001), Rrid059A redesigned (Hotz et al. 2001 and table 1: forward primer redesigned to extend the fragment amplified by 177 base pairs), Re1CAGA10 (Arioli 2007 cap. 4), RlCA1a27, ReGA1a23, Rrid169A and Rrid135A (table 1). Both forward and reverse primers appeared in 0.1 µM (or rarer 0.2 µM) in the PCR. Of the forward primers, 8-40% were color labeled with FAM, VIC, NED or PET. PCR 1 was given 15 min of initial denaturation at 95° C, 30 cycles of 30s at 94° C, 90s at 57° C and 60s at 72° C and a final extension of 30 min at 60° C. PCR 2 was run similarly, but with 31 cycles with 60° C in stead of 57° C. 0.7µl of the PCR products were run on an ABI 3730 Avant capillary sequencer with internal size standard (GeneScan-500 LIZ) and the alleles were scored with the Genemapper software (Applied_Biosystems 2004).

Genotyping

All samples were analyzed with 18 primer pairs amplifying loci in either the L genome, the R genome or both. The 18 primers were scored at a total of 13 loci in each genome. With some primers, genome specificity changed slightly with PCR conditions, i.e. typically monomorphic L-specific alleles could arise or disappear beside the R allele(s) according to

336 annealing temperature or primer concentrations. However, monomorphic loci conveyed no
337 information of importance for the present study, and the choice of scoring or leaving out
338 particular loci for technical reasons would not bias the data on homozygosity/heterozygosity
339 which was the focus of this study.

340 All alleles scored were specific to either the L or the R genome. Allele specificity was
341 confirmed in *P. lessonae*, *P. esculentus* and *P. ridibundus* from Estonia, Latvia and Lithuania
342 (unpublished data), in non-hybrid LL and RR offspring from the crossings and through the
343 distribution of L and R specific alleles on LLR, LR and LRR frogs. Preliminary data from
344 German and Swiss samples indicated, however, that in these more southern populations with
345 higher genetic polymorphism, certain alleles were not genome-specific.

346 Four of the primer pairs (Res16, RlCA1b5, Ca1b6 and Ga1a19) amplifying both L and
347 R specific alleles were used to distinguish LLR, LR and LRR frogs by dosage effect, i.e. by
348 the relative intensities (peak heights) of the L and R alleles amplified (see Christiansen 2005).
349 L:R peak heights were evaluated separately per 96-well PCR, both per locus and per allele
350 combination within that locus. The great majority of the L:R peak height ratios clustered into
351 discrete groups corresponding to the LLR, LR and LRR genotypes. Samples producing
352 intermediate or extreme L:R ratios were subjected to repeated PCR analyses until each of the
353 four dosage effect loci clearly signaled LLR, LR or LRR. Assignment to LLR, LR or LRR
354 was thus determined independently at four loci. In non-hybrid offspring (LLL, LL, RR, RRR)
355 the peak height ratios of heterozygous L or R loci were used to determine ploidy in the same
356 way as just described. Not all loci and allele combinations proved diagnostic, but most did.

357 Samples that repeatedly gave conflicting results on genotype, i.e. had extra or missing
358 alleles at particular loci, were classified as mixed genotypes. Mixed genotypes, constituted
359 3.6% of the crossing experiment offspring and 2.1% (2.7% inclusive null alleles, see below)
360 of the natural pond samples and were excluded from data sets where the relevant loci could
361 not be scored unambiguously.

Null alleles, i.e. alleles missing according to the overall ploidy of the individual, can be a nuisance in population genetics, because in high frequencies they bias estimates of allele frequencies and heterozygosity. However, in this study, they were generally not a problem, as they were often directly detectable and occurred in low frequencies only. The adults used for crossings carried no problematic null alleles, as the analyses were made on the loci where they were heterozygous for real alleles. Spontaneously missing alleles in mutant crossing experiment offspring, as well as null alleles in the frogs from the natural populations, were all directly detectable at the four dosage effect loci, and on average half of them were unmasked and detectable in a hemizygous state at the remaining loci. For example, a null allele at an L locus without dosage effect would be masked in LLR frogs but unmasked in LR and LRR frogs. Individuals with detected null alleles were handled as mixed genotypes described above. Only in two ponds was the same locus found missing in more than two frogs (i.e. six and eight frogs respectively), indicating that undetected null alleles could occur at potentially problematic frequencies in these ponds. In one of the two ponds, the entire locus was therefore recoded as missing data. In the second pond, all individuals were hemizygous at that locus, so that the null allele could always be detected. It was therefore coded as a real allele. For determining LLR and LRR proportions in the ponds, mixed genotypes were assigned to the most similar euploid genotype.

Statistics: crossings

The crossings yielded data from on 30 triploid frogs for segregation and linkage analyses. For males, the analyses were based on 19-58 (mean 41) offspring and for females on 30-86 (mean 66) offspring, as females were on average mated to more partners than males.

Non-random segregation would indicate selection during the experiment or unexpected genetic mechanisms. To check for random segregation at the heterozygous loci in the parents, offspring allele frequencies were tested with Chi-square tests for homogeneity

with Yate's correction for continuity (Fowler and Cohen 1992). To correct for multiple tests (n = 55 L and 57 R loci), sequential Bonferroni correction of the P values was calculated according to Holm (1979) in the program MacBonferroni (Watkins 2002).

Linkage analysis involves analysis of the inheritance pattern at two loci that are heterozygous in a parent (e.g. Aa+Bb). *Without* recombination, all pairs of loci should show complete linkage, i.e. only two of the parent's allele combinations should be observed in the offspring (e.g. A+B and a+b). In contrast, *with* recombination all four possible combinations should be found in the offspring (A+B, a+b, A+b and a+B) in approximately equal proportions of 0.25. Intermediate results, where the recombinant allele combinations (A+b and a+B) are significantly less frequent than the parental ones (A+B and a+b), would indicate reduced recombination and would be hard to explain if deriving from the majority the locus pairs. However, a few locus pairs must, by chance, be expected to have reduced or no recombination, due to physical linkage. Linkage was investigated with 2x2 Chi-square tests with Yate's correction for continuity (Fowler and Cohen 1992) for every pairwise combination of loci that were heterozygous in the parent.

Statistics: natural populations

The rate of recombination is not easily measured directly. Instead, linkage disequilibrium between multiple genetic markers was used for an indirect measure, as recombination and linkage disequilibrium should be negatively related (see the discussion). Pairwise and multilocus linkage disequilibria in natural populations were calculated as $\bar{r}_d$, as recommended by Halkett *et al.* (2005). $\bar{r}_d$ is an index of association adjusted for unequal sample size, calculated by the program Multilocus (Agapow and Burt 2001). First, L and R loci were divided into separate datasets. Then, the two homospecific allele sets in triploids were split up into haploid data by recoding all but one randomly chosen heterozygous locus into missing data. Recoding heterozygous loci into missing data is also how Multilocus

414 handles diploid data, according to the documentation file. Calculations were based on 20-71
415 (mean 37) haplotypes in Danish ponds and 56-110 (mean 78) in Swedish ponds. One pond
416 was excluded from the L and another from the R data set because less than our predefined
417 minimum of 20 haploid genotypes had been sampled. Two further ponds were excluded from
418 the L data and eight from the R data because no or only one locus was polymorphic. After
419 that, the genomes had 2-11 variable loci (mean 3.8 for the L and 5.2 for the R), i.e. loci with
420 at least 5 undeleted copies of an alternative allele.

421 Pairwise $\bar{r}_d$ was calculated in order check for locus pairs producing $\bar{r}_d$ values
422 differing significantly from the mean $\bar{r}_d$ of the remaining pairs, when tested pairwise (locus
423 pair in question vs. mean of remaining locus pairs) over all ponds. This pairwise within-pond
424 approach was necessary because overall linkage was expected to differ between ponds.
425 Significantly elevated linkage disequilibria could suggest physical linkage between the loci in
426 question, whereas linkage disequilibria lower than the mean would be difficult to explain.

427 Multilocus $\bar{r}_d$ were calculated for each genome in each pond to test the predicted
428 correlations between recombination and population structure outlined in the introduction. All
429 linear regressions, correlations and t-tests were performed in SPSS (2004). The L and R
430 slopes from the linear regressions were subjected to a test for difference between two
431 regression lines (Fowler and Cohen 1992).

432 The expected relationships between linkage disequilibrium and population structure
433 could be obscured by strong between-pond variation in the forces responsible for linkage
434 disequilibrium, i.e. founder effect, drift, migration and ecological selection on linked loci. If
435 these forces affect the L and R genomes to a similar extent, the magnitude of this problem
436 might be revealed by the degree of correlation between linkage disequilibrium in the L and R
437 genomes in the ponds. To test for such pond-specific effects, we correlated $\bar{r}_d$ values for the
438 L and R genome.

Genetic diversity varied between ponds and was generally lower in the L-specific than the R-specific markers. To investigate whether the estimates of multilocus linkage disequilibrium were affected by this variation in genetic diversity, we tested for correlation between $\bar{r}_d$ and genetic diversity measured as expected heterozygosity summed over all loci per genome. Expected heterozygosity was for each locus calculated as $H_E = 1 - (al_1^2 + al_2^2 + al_3^2\ldots)$ from allele frequencies (al_1, al_2, al_3 etc) computed by the software, SPAGeDi (see below).

As mentioned above, all but one of the heterozygous loci in triploid frogs had to be excluded for the constructing haplotypes for calculating $\bar{r}_d$. This affected the R genome the most, as its higher genetic diversity resulted in many R-heterozygous LRR frogs. Ponds rich in LRR frogs could thus theoretically have lower $\bar{r}_d$ values as a result of the lower resolution after the exclusion of the many heterozygous loci. To investigate whether $\bar{r}_d$ was affected by the resolution, it was tested whether the $\bar{r}_d$ values for the R genome were correlation with the number of hemizygotes (LLR and LR which had no loci excluded) in the sample they were calculated from.

To investigate inbreeding and population structuring, F_{IS}, F_{ST} and F_{IT} were calculated in the program SPAGeDi (Hardy and Vekemans 2002), which accepts a mixture of different ploidy levels. These F statistics were calculated for each genome separately so that with respect to the L genome, LLR provided diploid data while LR and LRR provided haploid data. Similarly, LLR and LR gave haploid R data while LRR gave diploid R data. Excluding all haploid data from the analyses had very little effect on the results, though.

Results

Crosses

Recombination data was obtained from 7 LLR females, 10 LLR males, 7 LRR females and 6 LRR males. Due to multiple heterozygosity, most individuals provided data for several

pairwise locus combinations. The LLR frogs provided recombination data for a total of 18 out of 21 possible pairwise combinations of 7 polymorphic L loci, and the LRR frogs for 47 of 66 possible combinations of 12 polymorphic R loci. All heterozygous loci in these triploids demonstrated random segregation, i.e. none of the allele proportions differed significantly from 0.5 at the 0.05 significance level after sequential Bonferroni correction performed within each genome separately. All triploids produced three or four gamete types per locus pair, corresponding to the two parental types and one or both recombinant types. All triploids thus recombined all their loci, and only for one locus pair were not all four gamete types present.

The uncorrected P values for the Chi-square tested frequency distributions of the four possible gamete types per locus pair are shown in Fig. 2. As parental and recombinant gametes were indistinguishable because the genotypes of the parents of the frogs crossed were unknown, insignificant P value deviations from zero do not necessarily imply reduced recombination. Insignificant P value would also have resulted from randomly derived excess of recombinant gametes and from uneven allele frequencies within the expected numbers of parental and recombinant gametes. When considered individually, the -log(p) values exceeding 1.30 were significant at the 0.05 level. After within-genome sequential Bonferroni correction for the 65 tests in the L genome and the 91 tests in the R genome, however, only four P values were significant. This indicates that the great majority of locus pairs were unlinked and freely recombined.

The four locus pairs showing significant linkage occurred in four different frogs (represented by four filled symbol types in Fig. 2) that all produced equilibrium offspring frequencies at their remaining locus pairs. The linkage was therefore rather a property of the loci than of the frogs involved. Unfortunately, replicate data was not obtained for the three locus pairs giving the most significant P values in this study, but the pair with strongest linkage, Re1CAGA10 + RlCA18 (L genome), was the same pair for which Arioli (2007 cap. 1) found no recombination. From the 40, 0, 0, 38 gamete frequency distribution in that and the

20, 0, 3, 23 gamete frequency distribution in the present study, it can be inferred that that Re1CAGA10 and RlCA18 are linked, i.e. situated closely together on the same chromosome. Ca1b6 + Ga1a19 (R genome) had the offspring type distribution 10, 33, 18, 8 and Rrid169 + Rrid059A (R genome) had 36, 16, 7, 27. These locus pairs thus appear weakly linked, but replicate crossings would be needed to confirm linkage. Re2GAGA3 + Rrid135A appeared significantly linked in one female with gamete frequency distribution 12, 13, 20, 17, but unlinked in three other females. Overall, therefore, these two loci appear unlinked. Actually, a mutation happened in the germ line of this female so that some of her offspring had a new allele at locus Re2CAGA3. A rare allele at another locus confirmed that these offspring were indeed hers. The offspring with the new allele were excluded from the analyses involving Re2CAGA3, but when included by pooling the new and the lowest-frequency maternal allele, from which it most probably mutated, all four P values for locus pairs including Re2CAGA3 dropped substantially and the significant value became clearly non-significant.

Nearly significant P values appeared for several other locus pairs, but also here replicates raised the average for these loci to well above the 0.05 level, rendering no overall indication of linkage. Males and females did not have significantly different mean P values (male mean = 0.356, female mean = 0.285, t-test, $t_{154} = 1.606$, P = 0.110). Many species, probably including *P. esculentus* (Burt et al. 1991), have lower crossing-over rates in males than in females, but the present data set can neither confirm or disprove this for *P. esculentus*.

Natural populations

Triploids were found in all 55 ponds investigated, and both kinds (LLR and LRR) were found in 82% of the ponds. The proportion of LLR varied from 0-100% while that of LRR varied from 0-86% in the pond samples (Fig. 3). Of the 2296 frogs genotyped, only 0.2% were non-hybrid. These were 5 LL from two Swedish ponds. Multilocus linkage disequilibrium, measured as $\bar{r}_d$ on a scale from zero to one, averaged 0.01 in the L genome and 0.11 in the R

517 genome, indicating that both genomes were well recombined in the majority of the natural
518 populations. Mean $\bar{r}_d$ in the R genome was, however, significantly higher than in the L
519 genome (t-test, $t_{111} = -3.819$, $P < 0.001$).

520 Multilocus disequilibrium in the L genome showed no relation with the proportion of
521 LLR individuals (linear regression: $F_{1,61} = 2.269$, $P = 0.137$, $r^2 = 0.036$). In contrast,
522 multilocus disequilibrium in the R genome was negatively associated with the proportion of
523 recombining LRR frogs among the R gamete-producing LR and LRR frogs (linear regression:
524 $F_{1,54} = 9.034$, $P = 0.004$, $r^2 = 0.143$, slope = -0.214). These results were thus fully in
525 accordance with the expectations. The slopes of the L and the R regressions were, however,
526 not significantly different ($t_{115} = 1.440$, $P = 0.153$).

527 The multilocus linkage disequilibria ($\bar{r}_d$) in the L and the R genomes were not
528 positively correlated within ponds (Fig. 4). In fact, they were significantly negatively
529 correlated (Pearson correlation: $r_{55} = -0.374$, $P = 0.005$); even excluding the L outliers far left
530 and far right in Fig. 4. This indicates an absence of strong pond-specific effects affecting $\bar{r}_d$
531 in the L and R genome simultaneously.

532 There was no correlation between $\bar{r}_d$ and genetic diversity, measured as the expected
533 heterozygosity summed over all loci (Pearson correlation for L and R data pooled: $r_{119} =$
534 0.013, $P = 0.889$). The significant difference in mean multilocus disequilibrium between the L
535 and the R genome can therefore not be explained by lower polymorphism in the L specific
536 microsatellite loci, but only by differences in recombination rates. The $\bar{r}_d$ values for the R
537 genome showed also no correlation with the number of hemizygotes in the sample they were
538 calculated from (Pearson correlation: $r_{55} = 0.016$, $P = 0.904$). The significant relation between
539 LRR/(LR+LRR) and $\bar{r}_d$ in the R genome in Fig. 3b can therefore not be explained by
540 exclusion of heterozygous loci in LRR frogs, but must be attributed to differences in
541 recombination rates.

An analysis of pairwise $\bar{r}_d$ values showed that only two locus pair had $\bar{r}_d$ values differing significantly from the mean pairwise $\bar{r}_d$ of the remaining locus pairs in the same ponds (28 L and 63 R, paired t-tests with sequential Bonferroni correction within each genome separately). These two locus pairs (L loci Res20 + Re1CAGA10 and the R loci RlCA1b5 + Rrid064A) both had significantly lower $\bar{r}_d$ than the remaining loci. Thus, most locus pairs gave similar results within ponds and none gave elevated values suggesting linkage. In spite of the tight linkage in the crossing experiment, pairwise $\bar{r}_d$ for Re1CAGA10 + RlCA18 was not significantly different from the mean, even without Bonferroni correction (paired t-test: $t_9 = 1.311$, $P = 0.222$). The same applies to the two potentially linked locus pairs (Ca1b6 + Ga1a19: $t_{10} = -0.654$, $P = 0.528$; Rrid169 + Rrid059A: $t_{22} = 1.261$, $P = 0.221$). Therefore, these three (potentially) linked locus pairs were not excluded from the analyses of natural populations.

Global F_{IS} was very low in both the L and R genome, i.e. -0.007 and -0.008, respectively, indicating random mating within ponds. Global F_{ST} values were rather high, i.e. 0.4561 and 0.6156 in the L and R genome, respectively, indicating much genetic structure among ponds, which is in accordance with the expectations for a low-mobility animal. As a consequence of the low F_{IS}, F_{IT} was very similar to F_{ST} for both genomes.

Discussion

Recombination was demonstrated in all 30 frogs tested in the crossing experiment including both males and females of both LLR and LRR. As a consequence of such triploid-mediated recombination, natural populations were found to have low multilocus linkage disequilibria. In agreement with predictions from the asymmetrical propagation of L and R genomes in the all-hybrid populations, L genomes were generally fully recombined while R genomes were recombined according to the proportion of LRR triploids. The unique all-hybrid populations of *P. esculentus* are thus functionally sexual; actually, they represent an obligate symbiosis of

two independent, functionally sexual genomes: the L and the R genome. Below, we will first describe the genetic mechanisms underlying these results and then outline the evolutionary, conseptual and conservation-political implications for all-hybrid populations and hybridogenetic taxa.

Recombination in all-hybrid populations

In normal meiosis, the combined effects of random segregation of chromosomes and chromosomal crossing-over assure equal proportions of parental and recombinant gametes for most locus pairs. Reduced recombination rates due to physical linkage are, however, observed between loci situated so closely together on the same chromosome that there is small probability of crossing-over between them. A random sample of genetic markers for any kind of organism might thus include a small proportion of linked loci. *P. esculentus* has 13 chromosomes per L or R set (e.g. Koref-Santibanez and Günther 1980). The physical locations of our microsatellite loci on these chromosomes are unknown, but the results from the crossing experiment suggested linkage between three of the 65 locus pairs investigated. Loci Re1CAGA10 and RlCA18 showed strong linkage in a male crossed by us as well as in one crossed by Arioli (2007 cap. 1); thus it can be inferred that these two loci are situated close together. The apparent linkage of the two remaining locus pairs in this study was weaker and assessed in only one frog each, so that linkage should not be concluded without further verification. This discovery of one to three linked loci does not suggest variation in recombination rates among individuals, as the three frogs with apparently linked loci had full recombination at their remaining locus pairs investigated.

Selection took place in the crossing experiment, as dead and sick-looking tadpoles were not reared, and 10% of the offspring chosen for rearing eluded genotyping – mainly by dying. Only selection on the interaction of non-neutral loci linked to our markers could, however, have affected the recombination results. Any such interaction effects were reduced

by crossing every parent to several mates of different genotypes. As no significant bias in the segregation at any single locus was detected, bias of locus combinations by selection is unlikely. Furthermore, selection on the interaction of linked non-neutral loci would most likely bias the results towards less recombination, so it would not undermine the conclusion of recombination.

Unlike linkage, linkage disequilibrium can arise between loci without physical associations. Linkage disequilibrium, measured as $\bar{r}_d$ in the natural populations, is the net result of generating and deteriorating forces. Linkage disequilibrium-generating forces include founder effect, migration, drift, inbreeding and selection on linked genes, called hitchhiking (Hedrick 2005). In clonal organisms, the entire genome hitchhikes with positively selected genes. The deteriorating force is recombination. Linkage disequilibrium is a negative linear function of recombination rate per generation, with half of the disequilibrium disappearing per generation at 100% recombination (Hedrick 2005). Provided that $\bar{r}_d$ is a good measure of linkage disequilibrium, that LRR/(LR+LRR) was a fair substitute for recombination frequency in the ponds, and that disequilibrium-generating forces did not depend on population structure (e.g. on LRR/(LR+LRR)), linear relationships were therefore expected in Fig. 3.

With low $\bar{r}_d$ irrespective of population structure in the L genome (Fig. 3a) and a negative relationship between $\bar{r}_d$ and recombining triploids (LRR/LR+LRR) in the R genome (Fig. 3b), the expectations outlined in the introduction were met. According to the model by Som and Reyer (2006a), L genomes spend 2/3 of their generations in LLR frogs and 1/3 in LR frogs, which means that they are recombined two out of three generations. The empirical data from the present study shows that this recombination rate of 2/3, whatever the type and strength of linkage disequilibrium-generating forces in the natural populations, is sufficient to reduce $\bar{r}_d$ values to around zero (mean $\bar{r}_d = 0.01$ on the scale from zero to one). For the R genome, no theoretical model is available. Before a reliable model can be made, more

620 empirical data on the ratio of R and LR gametes produced by LR females and LR males is
621 needed, as this ratio is important for population dynamics and has been shown to vary
622 strongly between individuals and locations (Tunner and Heppich-Tunner 1991; Polls Pelaz
623 1994; Mikulícek and Kotlík 2001; Rybacki and Berger 2001; Christiansen et al. 2005; Arioli
624 2007 cap. 1; Jakob 2007 cap. 5). Central to such a model is also the question of why
625 populations vary in structure. Although it is commonly accepted that population structure of
626 *P. esculentus*, *P. lessonae* and/or *P. ridibundus* depend on ecological components (Pagano et
627 al. 2001; Holenweg Peter et al. 2002; Plötner 2005), attempts to identify the ecological
628 components determining population structure in Swedish all-hybrid populations were so far
629 rather inconclusive (Jakob 2007 cap. 3). In the absence of theoretical models, it was not
630 known what level of linkage disequilibrium to expect in the R genome of natural populations,
631 but the present empirical data show that it is generally low (mean $\bar{r}_d = 0.11$), although the
632 genetic signature of clonal reproduction was visible in certain populations with few LRR
633 frogs. In clonal populations of other organisms, $\bar{r}_d$ values have been found to be considerably
634 higher than in the present study (e.g. Goyeau et al. 2007; Grundmann et al. 2008).
635 Unfortunately, no thorough studies on multilocus disequilibrium in the *R. esculenta* LE or RE
636 system have been conducted yet.
637 The variation not explained by the linear relations in Fig. 3 is expected to derive from
638 three main sources. 1) Error on the estimate of $\bar{r}_d$ from a random sample of 17-86 (mean 35)
639 individuals. 2) Error on the estimate of population structure, e.g. LRR/(LR+LRR), from the
640 same random sample and between-pond-variation in the ratio of R gametes from LR frogs. If
641 the proportion of R gametes made by LR frogs varies between ponds, this will add further
642 noise. 3) Between-pond variation in the strength of the various disequilibrium-generating
643 forces listed above. The combined effects of these three sources explain the rather large
644 variation for the R genome in Fig. 3b. For the L genome, population structure (source 2)
645 should have no relevance, however. Furthermore, if the recombination rate is so high that it

646 always overpowers the local disequilibrium-generating forces (source 3), as seems to be the
647 case in the L genome, variation comes only from the error on the estimate of $\bar{r}_d$ (source 1).
648 This explains the relative low variation in Fig. 3a. Unfortunately, disequilibrium-generating
649 forces are difficult to measure. The only disequilibrium-generating force, we could measure in
650 this study was inbreeding. The low F_{IS} values obtained indicated random mating, so that
651 inbreeding would have little effect on $\bar{r}_d$. We did, however, test for those pond-specific
652 effects that affect the L and R genome similarly. The lack of a positive correlation between
653 $\bar{r}_d$ in the L and R genome across ponds (Fig. 4) indicates that such forces were absent. In
654 conclusion, the forces generating multilocus linkage disequilibrium in the natural populations
655 could not be indentified, but between-pond variation in their strength and composition did not
656 pose a problem in this study. On the contrary: the good match of observed with expected
657 relations in Fig. 3a and b shows that $\bar{r}_d$ can be a useful tool in studies of recombination.
658 The extreme positive outlier in Fig. 3a calls for a different explanation than those
659 given for residual variation. This explanation has to apply to the L genome only, as the high L
660 $\bar{r}_d$ value was not matched by a high R value (Fig. 4). Notably, in this pond, a null allele was
661 scored as a real L allele, because it did not pose a technical problem. As pairwise $\bar{r}_d$ values
662 were elevated for all locus pairs in this pond, the null allele cannot account for its outlier
663 status, however. Exclusion of the locus with the null allele reduced $\bar{r}_d$ to 0.29, i.e. the point
664 remaind an outlier although less extreme. A better explanation for the high $\bar{r}_d$ value can be
665 derived from the pond's extreme left position in the Figure. Although necessary for
666 reproduction, LLR frogs were absent from our sample of 23 adults. Also notable, although not
667 exceptional for this pond, was that the population appeared small with few males, which are
668 more often LLR than females. We could therefore speculate that the L genomes in the
669 sampled frogs derived from very few LLR ancestors. A linkage disequilibrium in the L
670 genome caused by such a bottleneck in LLR frogs would persist for several generations of
671 recombination.

672

673 **Evolutionary consequences**

674 Triploids are not restricted to all-hybrid populations, but have been found in various
675 population types in Germany (Günther 1975), Poland (Rybacki and Berger 2001) and France
676 (Regnier and Neveu 1986). The ability to make diploid eggs giving rise to triploid individuals
677 provides all these *P. esculentus* populations with genetic recombination and potential
678 reproductive independence - two important steps in the direction of speciation. Where hybrids
679 live sympatrically with parental species, they do not reproduce independently, however, but
680 interbreed with the parental species. Here, recombination by triploids might be of little
681 genetic importance to the hybrids, because they can be supplied with recombined genomes
682 from the parental species. In contrast, the all-hybrid populations of Denmark and southern
683 Sweden must rely on recombination in triploids only, as they are isolated from the nearest
684 parental populations by sea or large stretches of uninhabited land, and non-hybrid LL and RR
685 offspring only very rarely survive to sexual maturity (Christiansen et al. 2005; Jakob 2007
686 cap. 2 and the present study). Here, *P. esculentus* has truly accomplished the transition from a
687 clonal, gamete-dependent hybrid to an independent, sexually reproducing evolutionary unit.

688 Although the all-hybrid populations have a combination of clonal and sexual
689 reproduction, the low multilocus linkage disequilibrium values indicate that the loci of natural
690 populations were well mixed. Selection should thus have the whole range of genetic
691 combinations to work on, enabling beneficial, as well as harmful, mutations to be combined
692 for fast adaption to changing environments (Fisher 1930) and for purging of deleterious
693 mutations (Muller 1932). This hybridogenetic reproduction mode also ensures continuous
694 genetic variation as a defense against fast evolving parasites (Red Queen hypothesis,
695 Hamilton 1980), since the combination of recombined and clonal gametes result in unique
696 individuals. The all-hybrid populations thus seem to have all the advantages of sexual
697 reproduction, including a long-term evolutionary potential. The ability of fast adaption to

698 changing environments might, however, be of more importance for the survival of *P.*
699 *esculentus*, given that habitat loss and climate change increasingly threaten amphibians
700 worldwide (Stuart et al. 2004).

701 It remains to be analyzed to what extent all-hybrid *P. esculentus* populations can also
702 benefit from the clonal reproduction of diploids. In general, potential benefits of clonal
703 reproduction include the possibility to save the costs of producing males and the ability to
704 propagate favorable gene combinations (Otto and Gerstein 2006). In all-hybrid *P. esculentus*
705 populations, the theoretical offspring sex ratio is only slightly female biased which is in
706 agreement with the mean observed adult sex ratio in large surveys (Som and Reyer 2006b;
707 Jakob 2007 cap. 2 and the present study). Thus, only a few percent of the cost of males might
708 be saved. Recombination takes place after maximum one generation in the L genome (Som
709 and Reyer 2006a) and after one to a few generations in the R genome, suggesting that
710 favorable gene combinations are not be preserved for long, unless physically linked.
711 Therefore, the benefit that all-hybrid populations of *P. esculentus* can potentially derive from
712 the clonal component in their reproduction appears small - in contrast to cyclical
713 parthenogens, such as aphids, rotifers, water fleas that have successfully combined the
714 advantages of sexual and clonal reproduction (Innes and Singleton 2000).

715 With sexual reproduction, the death of newly formed non-hybrid LL and RR in the all-
716 hybrid populations is intriguing, because it cannot be attributed to clonal propagation of the
717 genomes, as in the LE system. In the LE system, RR die because recessive deleterious
718 mutations have become fixed in the clonally propagated R genome of the diploid LR hybrids
719 (Vorburger 2001a; Guex et al. 2002). These deleterious mutations were either acquired
720 through Muller's ratchet or were already present at hemiclone formation (Vorburger 2001a).
721 In all-hybrid EE populations, both genomes are regularly recombined in triploid individuals,
722 the L when in LLR and the R when in LRR. Hence, fixation of deleterious mutations by
723 Muller's ratchet is unlikely, yet fixation may still have occurred by other mechanisms, for

example founder effect. Fixation and low genetic diversity is certainly observed at microsatellite loci (Christiansen et al. 2005; Arioli 2007 cap. 4 and the present study). Explanations for how genetic diversity became and remained this low in spite of the presence of parental species just south of the German and north of the Swedish all-hybrid populations are, however, lacking.

P. esculentus most closely resembles the Iberian minnow, *Squalius alburnoides* (also called *Leuciscus*, *Rutilus* and *Tropidophoxinellus*, reviewed by Alves et al. 2001) of other hybridogenetic taxa known: both hybrids often form mixed populations of di- and polyploid hybrids and one or both parental genotypes. All-hybrid di- and triploid populations are, however, not known from *S. alburnoides*. In stead, tetraploids occur in many *S. alburnoides* populations and, in special habitats, tetraploids can constitute 73% of the mixed populations. These tetraploids have an even sex ratio, have normal meiosis, produce tetraploid offspring when mating with each other and appear to be reproductively isolated from other ploidy levels (Cunha et al. 2008). The discovery of these mainly tetraploid populations strongly suggests that meiotic hybridogenesis can act as a stepping stone to tetraploidization and ultimately to speciation. In *P. esculentus*, tetraploidy has so far only been found in very low frequencies in Swedish populations (Jakob 2007 cap. 2).

Given that recombination appears to be the rule in polyploid hybridogens and that polyploidy in hybridogenetic taxa appears to be more common than previously assumed, the prevailing view of hybridogens as clonally reproducing diploids may have to be changed. Should the discoveries of hybridogenetic breeding systems continue to increase, which is likely as more and more supposedly normal species are being genetically analyzed, this will also affect our perception of the importance of hybridization for speciation in animals.

Studies on hybrids are also relevant from a conservation point of view. Modern management concepts stress the importance of conserving “evolutionary significant units” (ESUs), i.e. populations representing significant adaptive variation; but how these units are to

750 be identified, is strongly debated (reviewed by Crandall et al. 2000). Hybrids, for instance, are
751 exempt from protection, because they do not seem to constitute independent evolutionary
752 lineages (Kraus 1995). While this may be true for F_1 progeny from many interspecific
753 matings, it is not true for parthenogenetic, gynogenetic and hybridogenetic taxa of hybrid
754 origin, which are capable of self propagation (Ranker and Arft 1994; Kraus 1995). This, plus
755 the finding that hybridogens like *P. esculentus* and *S. alburnoides* can form independent and
756 sexually reproducing populations, makes these organisms evolutionary significant units and
757 worthy of protection.
758

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1005

1006 ## Tables

1007 **Table 1.** Primer sequences not previously published.

Locus	Sequence 5' - 3'	Repeat	Genome specificity	Genbank ass. no.	Sequenced by
ReGA1a23	F: ATT GCT TTG GCA GTG AAG G R: TGA CAT CAC AGT GGG AGG AG	GA_n	L	EU445523	Garner *et al.*, Arioli and Jakob
RlCA1a27	F: CAA ATG GGT CAT CCA CAC C R: GTT CAA GGG GGT CGA AAT AC	CA_n	L	EU445522	Garner *et al.*
RlCA2a34	F: GCT CCA TGC CAA AAG TCT TC R: TTG GGT ATG ATA CTA CAA GCT ATG C	GT_n	L +R[1]	EU445521	Garner *et al.*
Rrid059A redesigned	F: TTG GAG ACA GAC TTC CGT AGG	CA_n	L[1]+R	FJ024048	Hotz *et al.*
Rrid064A	F: TGT ACG GGC CTT TAG ACT GG R: AAC TTT TTG AAG GCC CCT TG	GT_n… …TA_nGT_n	R	EU445524	Hotz *et al.*
Rrid135A	F: TCT TTT GTT TTA GCG CAC CT R: CTG CCC GTC TAA GCA AGT GT	CA_nTA_n	R	EU445526	Hotz *et al.*
Rrid169A	F: CGG AAC TCC GCT TTA ATC AC R: CCC ATG TTG TCG TTG AGC TA	TA_n…CA_n	R	EU445525	Hotz *et al.*

1008 [1] monomorphic in this genome

1009

1010 ## Figure legends

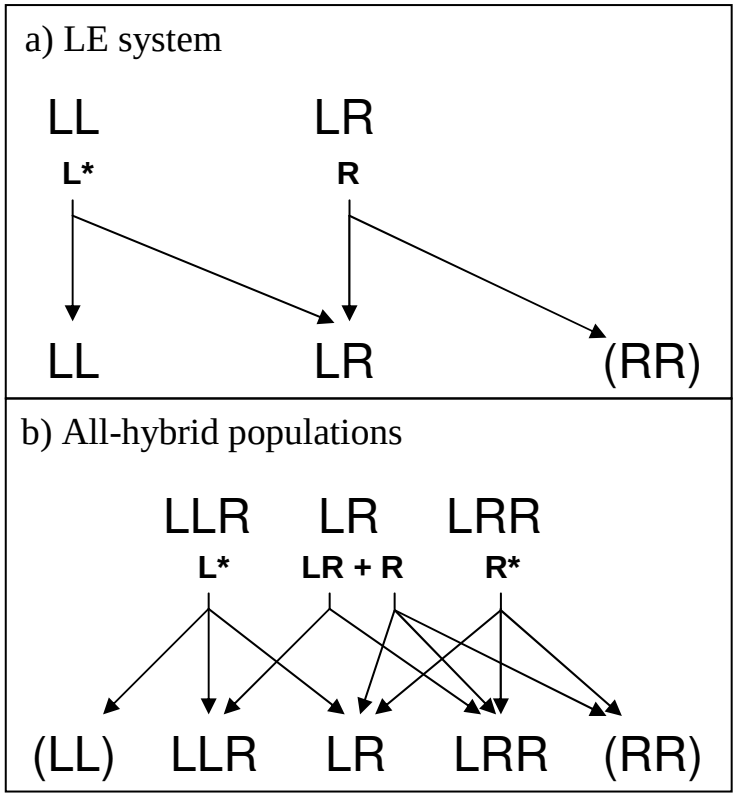
1011 **Figure 1.** Adults, gametes and offspring of a) the LE system with *P. lessonae* and *P.*
1012 *esculentus* and b) all-hybrid populations of *P. esculentus*. * denotes gametes that could be
1013 recombined. Non-hybrid offspring from intraspecific *P. esculentus* matings are in parenthesis

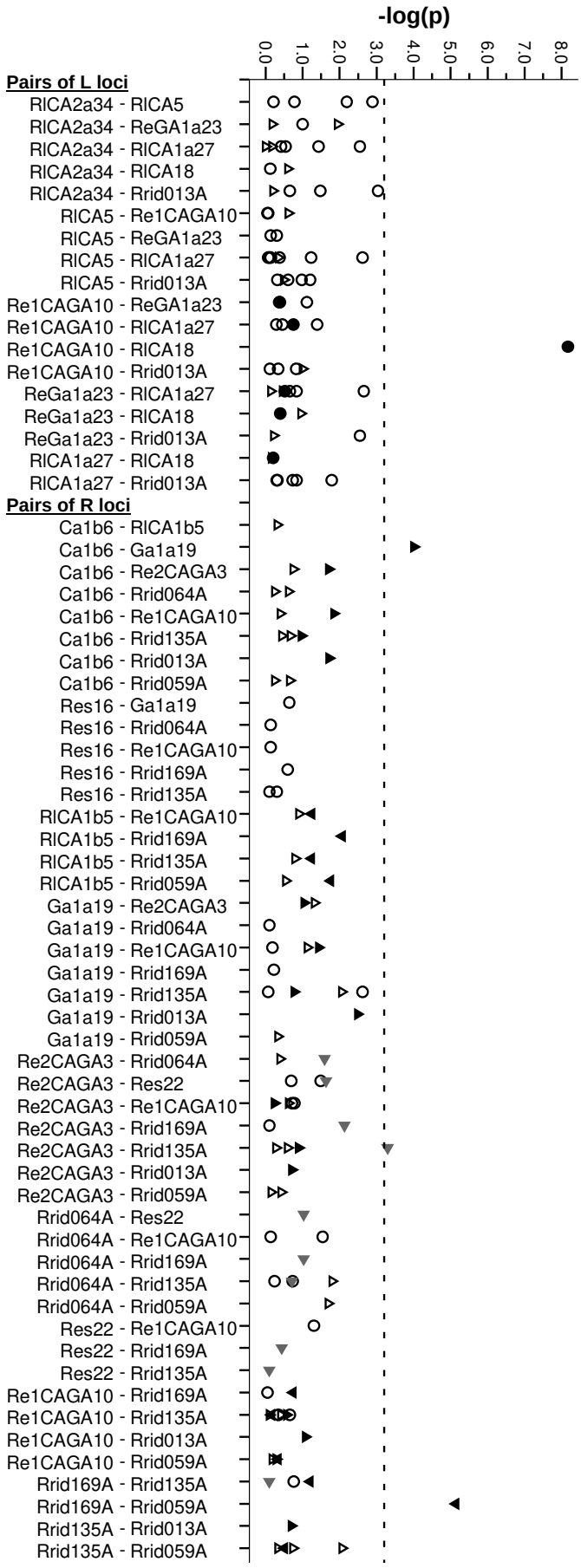
because they typically die before reproductive maturity. Note that in the LE system, the R genome is never recombined and the L genome is provided anew in every generation by *P. lessonae*. In the all-hybrid populations, both L and R genomes are supplied by hybrids and would regularly undergo recombination, if triploids have meiotic hybridogenesis.

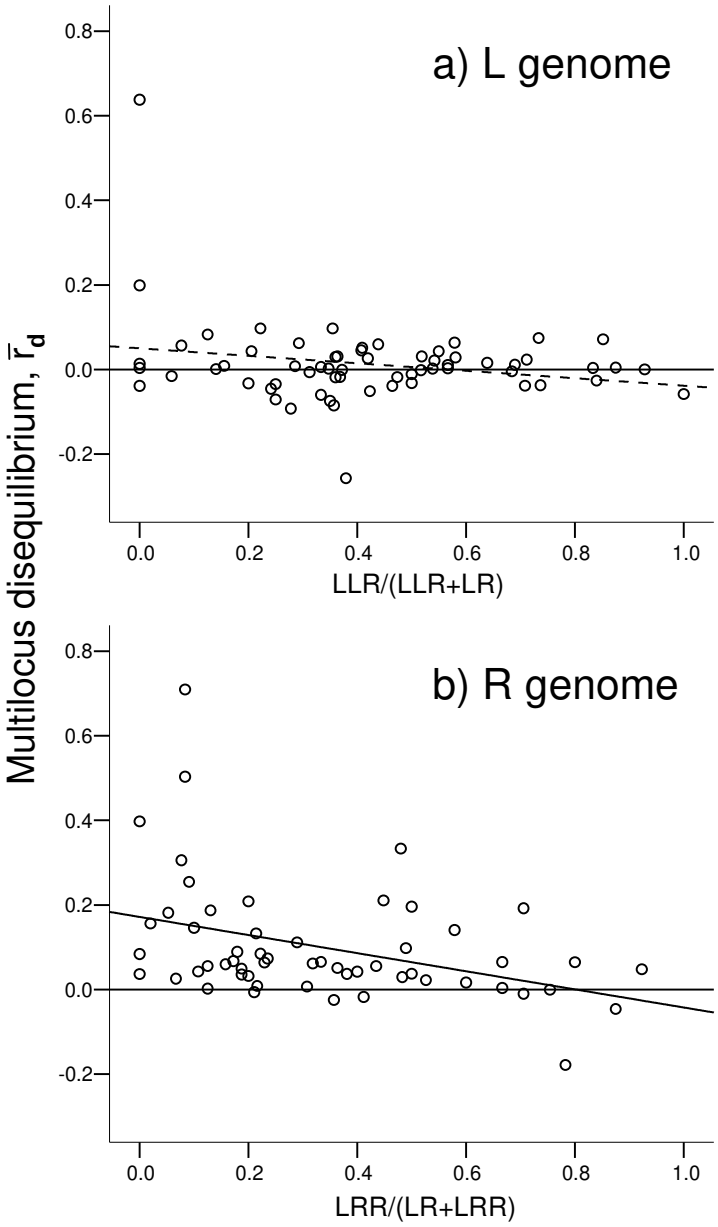
Figure 2. Linkage analysis of various locus combinations in crossing experiment with triploid *P. esculentus*. The symbols represent $-\log_{10}$ P values from Chi-square tests of the frequency distributions of the four potential (two parental and two recombinant) gamete types produced. Circles = males, triangles = females. Most individuals were heterozygous at several loci and therefore contributed data for several locus pairs. Each point left of the dashed line indicate a freely recombined locus pair in a frog. Points right of the dashed line indicate significant linkage at the 0.05 level after sequential Bonferroni-correction within each genome separately. Filled symbols (circles and triangles pointing right, left and down) identify all P values derived from the four individuals that each gave a significant P value. The female identified by grey triangles pointing down had a mutation at Re2GACA3 in her germ line which she passed on to some of her offspring.

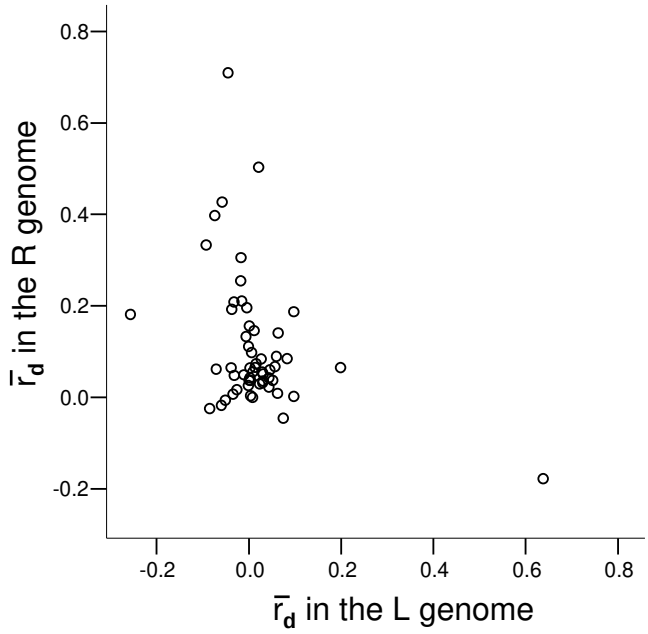
Figure 3. Multilocus linkage disequilibrium, $\bar{r}_d$, as a function of the proportion of frogs producing recombined gametes in 66 *P. esculentus* populations from Denmark and Sweden. a) $\bar{r}_d$ in the L genome vs. recombining LLR frogs of the total number of frogs propagating L genomes (LLR+LR). Linear regression line dashed because non-significant. b) $\bar{r}_d$ in the R genome vs. recombining LRR/total R-propagating frogs; regression significant. $\bar{r}_d$ is an index of association adjusted for unequal sample size.

Figure 4. Multilocus linkage disequilibrium ($\bar{r}_d$) in the L vs. the R genome in 56 ponds.


a) LE system
LL
LR
L*
R
LL
LR
(RR)
b) All-hybrid populations
LLR
LR
LRR
L*
LR + R
R*
(LL)
LLR
LR
LRR
(RR)


-log(p)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Pairs of L loci
RlCA2a34 - RlCA5
RlCA2a34 - ReGA1a23
RlCA2a34 - RlCA1a27
RlCA2a34 - RlCA18
RlCA2a34 - Rrid013A
RlCA5 - Re1CAGA10
RlCA5 - ReGA1a23
RlCA5 - RlCA1a27
RlCA5 - Rrid013A
Re1CAGA10 - ReGA1a23
Re1CAGA10 - RlCA1a27
Re1CAGA10 - RlCA18
Re1CAGA10 - Rrid013A
ReGa1a23 - RlCA1a27
ReGa1a23 - RlCA18
ReGa1a23 - Rrid013A
RlCA1a27 - RlCA18
RlCA1a27 - Rrid013A
Pairs of R loci
Ca1b6 - RlCA1b5
Ca1b6 - Ga1a19
Ca1b6 - Re2CAGA3
Ca1b6 - Rrid064A
Ca1b6 - Re1CAGA10
Ca1b6 - Rrid135A
Ca1b6 - Rrid013A
Ca1b6 - Rrid059A
Res16 - Ga1a19
Res16 - Rrid064A
Res16 - Re1CAGA10
Res16 - Rrid169A
Res16 - Rrid135A
RlCA1b5 - Re1CAGA10
RlCA1b5 - Rrid169A
RlCA1b5 - Rrid135A
RlCA1b5 - Rrid059A
Ga1a19 - Re2CAGA3
Ga1a19 - Rrid064A
Ga1a19 - Re1CAGA10
Ga1a19 - Rrid169A
Ga1a19 - Rrid135A
Ga1a19 - Rrid013A
Ga1a19 - Rrid059A
Re2CAGA3 - Rrid064A
Re2CAGA3 - Res22
Re2CAGA3 - Re1CAGA10
Re2CAGA3 - Rrid169A
Re2CAGA3 - Rrid135A
Re2CAGA3 - Rrid013A
Re2CAGA3 - Rrid059A
Rrid064A - Res22
Rrid064A - Re1CAGA10
Rrid064A - Rrid169A
Rrid064A - Rrid135A
Rrid064A - Rrid059A
Res22 - Re1CAGA10
Res22 - Rrid169A
Res22 - Rrid135A
Re1CAGA10 - Rrid169A
Re1CAGA10 - Rrid135A
Re1CAGA10 - Rrid013A
Re1CAGA10 - Rrid059A
Rrid169A - Rrid135A
Rrid169A - Rrid059A
Rrid135A - Rrid013A
Rrid135A - Rrid059A


a) L genome
b) R genome
Multilocus disequilibrium, $\bar{r}_d$
LLR/(LLR+LR)
LRR/(LR+LRR)
0.8
0.6
0.4
0.2
0.0
-0.2
0.0
0.2
0.4
0.6
0.8
1.0


0.8
0.6
0.4
0.2
0.0
-0.2
$\bar{r}_d$ in the R genome
-0.2
0.0
0.2
0.4
0.6
0.8
$\bar{r}_d$ in the L genome